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Dissecting histone deacetylase role in pulmonary arterial smooth muscle cell proliferation and migration

Margherita Galletti [a,b,1], Silvia Cantoni [a,b,1,*], Filippo Zambelli [a,c], Sabrina Valente [b], Massimiliano Palazzini [b], Alessandra Manes [b], Gianandrea Pasquinelli [b], Antonello Mai [d], Nazzareno Galiè [a,b], Carlo Ventura [a,e]

[a] National Institute of Biostructures and Biosystems (NIBB), University of Bologna, Bologna, Italy
[b] Department of Experimental, Diagnostic and Specialty Medicine (DIMES), University of Bologna, Bologna, Italy
[c] Società Italiana Studi Medicina della Riproduzione (S.I.S.Me.R.), Reproductive Medicine Unit, Bologna, Italy
[d] Department of Drug Chemistry & Technologies, Sapienza University of Rome, Rome, Italy
[e] Stem Wave Institute for Tissue Healing (SWITH), Gruppo Villa Maria (GVM) and Ettore Sansavini Health Foundation – ONLUS, Lugo, Ravenna, Italy

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ABSTRACT

Pulmonary Arterial Hypertension (PAH) is a rare and devasting condition characterized by elevated pulmonary vascular resistance and pulmonary artery pressure leading to right-heart failure and premature death.

Pathologic alterations in proliferation, migration and survival of all cell types composing the vascular tissue play a key role in the occlusion of the vascular lumen. In the current study, we initially investigated the action of selective class I and class II HDAC inhibitors on the proliferation and migration of pulmonary artery smooth muscle cells (PASMCs) after exposure to Platelet Derived Growth Factor (PDGF). Class I HDAC inhibitors were able to counteract the hyperproliferative response to PDGF, reducing both proliferation and migration in PASMCs, while class II were ineffective. Selective silencing with siRNAs targeted against different HDACs revealed a major role of class I, and within this class, of HDAC1 in mediating PDGF-induced Akt Phosphorylation and Cyclin D1 (CycD1) expression. These results from these combinatorial approaches were further confirmed by the ability of a specific HDAC1 inhibitor to antagonize the PDGF action. The finding that HDAC1 is a major conductor of PDGF-induced patterning in PAH-PASMCs prompts the development of novel selective inhibitors of this member of class I HDACs as a potential tool to control lung vascular homeostasis in PAH.

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1. Introduction

Pulmonary Arterial Hypertension (PAH) is a rare and multifactorial condition characterized by elevated pulmonary vascular resistance and pulmonary artery pressure leading to right-heart failure and premature death [1,2].

The progressive increase of pressure load is due to a dramatic pulmonary arterial wall remodeling involving all cellular components of the vessel. Dysfunction in cell proliferation, migration, survival, inflammation and thrombosis lead to a thickening of the vascular walls and to a subsequent occlusion of the lumen [3]. Within many factors involved in these processes, platelet derived growth factor (PDGF) pathway plays a pivotal role in modulating Pulmonary Artery Smooth Muscle Cells (PASMCs) proliferation and migration [4,5].

Current treatments are only able to slow down the progression of PAH thus a real cure does not exist [6]. Furthermore, the mechanisms underlying the onset and progression of PAH have not been fully elucidated yet. For these reasons studies addressing the etiology of the disease and directed to identify new therapeutic targets are needed.

Histone Deacetylases (HDACs) remove the acetyl group from the amino termini of Lysine residues of histones and other various substrates including transcription factors, chaperones, as well as

* Corresponding author at: Laboratory of Experimental Cardiology, Pav.21 DIMES University of Bologna, Via Massarenti 9, 40138 Bologna, Italy.
Tel.: +39 0516363605; fax: +39 051344859.
E-mail address: silcant@gmail.com (S. Cantoni).
[1] These authors contributed equally to this work.

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many regulators involved in DNA repair, cell signaling or metabolism [7]. Within 18 mammalian family members the 11 known zinc-dependent HDACs are classified into 4 subgroups: class I (HDAC 1, 2, 3, 8), class IIa (HDAC 4, 5, 7, 9), class IIb (HDAC 6, 10) and class IV (HDAC 11) [8,9].

These enzymes are involved in many different biological and pathological processes: their role in cancer progression has long been established since 1998 [10] but these molecules have been associated also with Pulmonary Arterial Hypertension [11], diabetes [12], memory formation [13], Parkinson disease [14] and inflammation processes in Rheumatoid Arthritis [15]. Histone Deacetylase inhibitors (HDACis) have been developed to counteract the proliferation of cancer cells and showed promising results by inducing cell cycle arrest, apoptosis and anti-angiogenic effects [16]. Pan-HDACis also reduce cardiac hypertrophy in PAH rats and inhibit the "highly proliferative phenotype" of PDGF-treated vascular cells [11]; selective class I HDACis counteract hypoxia-induced cardiopulmonary remodeling in rats [17] and class II HDACis are involved in blocking the fetal gene reactivation in heart, reducing hypertrophy [18]. Among different classes, however, it is still not clear which specific HDAC is involved in promoting these pathological processes. In PAH abnormalities are found in both heart and lung cells. The need for "more" specific targets also arises from the fact that different HDACs among their class I have been reported to have distinct functions in heart and lungs: it is known indeed that the inhibition of class I HDAC reduces cardiopulmonary vessel remodeling, but the knockout of HDAC3 (belonging to class I) induces a hypertrophic phenotype in hearts [19].

We previously provide evidence that a pan-inhibitor of HDACs, Sodium Butyrate (NaBU), reduced the proliferation and migration induced by PDGF in PASMCs isolated from rats with monocrotaline (MCT)-induced PAH [20].

In the current study, we aimed at further dissecting the role of HDACs in these cells, and attempted to establish whether the proliferative/migratory action of PDGF may be executed through the intervention of multiple classes of HDACs, including class I and II, or it may conversely involve a single HDAC class, and in the affirmative, a targeted class member. For this purpose, we used a combinatorial approach encompassing the use of two novel hydroxamate derivatives MC1855 [21] and MC1575 [8], displaying specific inhibitory action toward class I and II HDACs, respectively, as well as the use of a specific inhibitor of class I HADC1. With this strategy, and by the aid of siRNAs specifically directed against each member of class I HDAC, we provide evidence that the PDGF effects on PAH PASMCs were primarily elicited through the activity of class I HDAC1.

2. Methods

2.1. Ethics statement

Animal use was approved by the Bioethics Committee of the University of Bologna, in compliance with Directive 2010/63/EU of the European Parliament. The study was conducted adhering to the institutions guidelines for animal husbandry regarding food space light temperature (23–25 °C).

2.2. Cell isolation and culture conditions

Adult male Sprague-Dawley rats (200–250 g in body weight; Harlan Laboratories; Indianapolis, IN, USA) were subjected to a subcutaneous injection of 60 mg/kg of MCT (Sigma–Aldrich; St. Louis, MO, USA) [20]. After 28 days, all rats were anesthetized in a CO_2/O_2 mixture and subsequently killed by cervical dislocation. Intrapulmonary arteries were isolated and cleaned of connective tissue. The isolation of PASMCs, from PAH-animals, has been performed using a modification of previously described method [22]. The tissue was digested at 37 °C for 20 min in DMEM containing collagenase type I, 250 U/ml (Sigma–Aldrich; St. Louis, MO, USA). Fetal Bovine Serum (FBS) 10% was added to stop the reaction and the digested pieces were placed into petri dish containing fresh complete medium. After reaching confluence cells were frozen. PASMCs were cultured in DMEM with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, 4 mM L-glutamine (all reagents from LONZA; Basel, Switzerland). PASMCs from the third to the fourth passages were used for all studies. To induce cell cycle synchronization a serum starvation was performed culturing cells at low percentage of serum (0.5% FBS) for 24 h. PASMCs were induced to proliferate and migrate by replacing starvation medium with fresh low serum medium containing 20 ng/ml PDGF (PeProtech; Rocky Hill, NJ, USA). MC1855 and MC1575 were synthesized in our laboratories at Sapienza University of Rome (Rome, Italy) as previously described [23], dissolved in DMSO and stored at −20 °C before use. NaBU and 4-(dimethylamino)-N-[6-(hydroxy amino)-6-oxohexyl]-benzamide (HDAC1-I) were purchased from Sigma–Aldrich (St. Louis, MO, USA) and Santa Cruz Inc. (Santa Cruz, CA, USA), respectively. The HDACis were added at the indicated concentrations concurrently with the PDGF, while the phosphatase inhibitor Calyculin A (CA), (Sigma–Aldrich; St. Louis, MO, USA), were administered 1 μM 1 h before the treatment. All experiments were repeated at least five times, unless otherwise mentioned.

2.3. MTT assay

PASMCs were seeded 3000 cells/cm^2 in 48-well plates and starved for 24 h. After 24 h of treatment MTT assay (Sigma–Aldrich; St. Louis, MO, USA) was performed to test cell growth. Cells were then incubated with MTT (20 μl/well of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in phosphate buffered saline [PBS]) for 3 h and lysed with the same volume of lysis buffer (10% SDS 10 mM NaOH), the absorbance was measured at 570 nm with a spectrophotometric plate reader.

2.4. Measurement of cell migration in vitro

Cells were seeded (15,000 cells/well in 24-well plates), starved for 24 h and cultured with treating medium for additional 16 h. The confluent cell monolayer was scraped with a pipette tip across the diameter of the well. After removal of cell debris by washing with PBS two times, cells were observed for 6 h. The wound area at the beginning of wounding and at the end of treatment was photographed respectively under an inverted phase-contrast microscope equipped with digital sight camera (Nikon; Tokio, Japan). The recovered surface area was calculated by subtracting the wound area at the end of treatment from the corresponding original wound area.

2.5. Immunofluorescence

Cycling cells expressing ki67 protein were identified using a single immunofluorescence staining. PASMCs were seeded on glass slides into 12-well plates at a density of 3000 cells/cm^2, starved and treated with the appropriate molecules in low-serum medium. After 24 h, cells were washed, fixed in 2% paraformaldehyde in PBS for 4 min at room temperature (RT) and permeabilized with 0.5% Tryton X-100 for 10 min After washes, the slides were treated with 1% bovine serum albumin (BSA), (Sigma–Aldrich; St. Louis, MO, USA) in PBS solution for 30 min at RT in a wet chamber to reduce non-specific staining and stained for 1 h at 37 °C with monoclonal antibody anti-ki67 (1:100, Novocastra, Leica Microsystems;

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